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Metabolic Activation of Organic Extracts from Diesel, Coke Oven, Roofing Tar, and Cigarette Smoke Emissions in the Ames Assay

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16. ABSTRACT The role of metabolic activation in the difference between a microbial and mammalian bioassays in the ranking of genotoxic potency of several environmental emissions was investigated. Although the relative potency in the Ames assay correlated well with the relative potency in mammalian cell and mouse skin for a series of automotive emissions (diesel and gasoline) this was not the case for the coke oven, roofing tar and cigarette smoke condensate [CSC] emissions. This study examined several parameters of the metabolic activation with Salmonella typhimurium TA98 including S9 concentration and a comparison of Aroclor-1254 induced with uninduced S9 from both rat and hamster liver. The diesel emissions sample was direct acting while the other samples required activation. The standard S9 concentration (approximately 1.25 mg protein/plate) also produced the maximum mutagenic activity. Induced S9s produced higher mutagenic activity than uninduced. The hamster S9 gave significantly higher mutagenic activities than rat S9 for the coke oven and CSC. Comparison of the relative potency of these four samples between the microbial (Ames), mammalian cell (mouse lymphoma) and tumor initiation activity was not significantly altered. These results suggest that the differences observed between the relative mutagenic activity of these emissions in the mammalian cell and microbial assays was not due to a lack of optimization of the S9 system but may be inherent in the different response of the indicator cells to different chemical classes.

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Metabolic Activation of Organic Extracts From Diesel, Coke Oven, Roofing Tar, and Cigarette Smoke Emissions in the Ames Assay

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Four environmental emissions samples were ranked by their genotoxic potency in several bioassays. Although the relative potency of a series of automotive emissions (diesel and gasoline) in the Ames assay correlated well with the relative potency in mammalian cell and mouse skin, this was not the case for the coke oven, roofing tar, and cigarette smoke condensate (CSC) emissions. This study examines the role of metabolic activation in determining the difference between a microbial and a mammalian bioassay in ranking the genotoxic potency of these environmental emissions. Uninduced and Aroclor 1254-induced S9 from both rat and hamster liver were compared as the metabolic activator in the Ames assay with *Salmonella typhimurium* TA98. The diesel emissions sample was direct-acting while the other samples required activation. The standard S9 concentration (only Aroclor-induced rat, approximately 1.25 mg protein/plate) also produced the maximum mutagenic activity. Induced S9s produced higher mutagenic activity than uninduced. The hamster S9 gave significantly higher mutagenic activities than rat S9 for the coke oven and CSC. The relative potency of these four samples was not significantly different between the microbial (Ames), mammalian cell (mouse lymphoma), and tumor initiation (mouse skin) assays. These results suggest that the differences observed between the relative mutagenic activity of these emissions in the mammalian cell and microbial assays was not due to a lack of optimization of the S9 system but may be inherent in the different response of the indicator cells to different chemical classes.

Key words: metabolic activation, diesel, coke oven, roofing tar, cigarette smoke condensate

INTRODUCTION

The *Salmonella*/mammalian microsome mutagenicity assay as developed by Ames et al [1975] is widely used as a short-term bioassay to screen complex environ-

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mental mixtures for mutagenic and potentially carcinogenic activity. The complex organics from extractable particles emitted by diesel engines were found to be mutagenic in the Ames assay [Wang et al, 1978; Huisingh et al, 1978], and shortly thereafter, a number of other combustion emissions were reported to be mutagenic in this assay [Claxton and Huisingh, 1980]. These observations were consistent with earlier findings that incomplete combustion products from coal tars and soot extracts were among the first recognized human and animal carcinogens [Kennaway, 1925; Cook et al, 1932; Cook and Kennaway, 1938]. The mutagenic activity of diesel emissions was compared to several complex emission sources including coke oven, roofing tar, and cigarette smoke condensate (CSC) emissions for which human cancer data were available [Claxton and Huisingh, 1980; Claxton, 1981]. All four of these emission samples were mutagenic in *Salmonella typhimurium* TA98 in the presence of metabolic activation. The diesel emissions were, in some cases, more mutagenic in the absence of metabolic activation. The relative mutagenicity of these environmental emissions in a battery of short-term microbial and mammalian cell bioassays has been compared to both the relative tumor initiating activity in mouse skin and the relative lung cancer risk in humans [Nesnow and Lewtas, 1981; Albert et al, 1983]. Although the relative potency in the Ames assay correlated well with the relative potency in mammalian cell and mouse skin for a series of automotive emissions (diesel and gasoline), this was not the case for the comparative emissions (coke oven, roofing tar, and CSC) [Nesnow and Lewtas, 1981].

Since these comparative samples either required or were enhanced by metabolic activation, it is possible that either the standard Aroclor-induced rat liver S9 activation system was not optimal or that the relative response in *S typhimurium* TA98 differs from the other bioassays owing to inherently different sensitivities to these different mixtures.

Several studies have compared the liver S9 fraction from such species as baboon, dog, rhesus monkey [Muller et al, 1980], guinea pig [Baker et al, 1980], or hamster [Raineri et al, 1981] with rat liver S9 as the metabolic activator in the Ames assay. Aroclor-induced hamster S9 was shown to be more effective than Aroclor-induced rat S9 in activating the aromatic amines 2-acetylaminofluorene and 2-aminoanthracene [Raineri et al, 1981] and diethyl nitrosamine [Prival and Mitchell, 1981], while the reverse was true with the polycyclic aromatic hydrocarbon benzo(a)pyrene [Raineri et al, 1981].

This study examined several parameters associated with the metabolic activation system that may effect the mutagenic activity and slope of the dose-response in *S typhimurium* TA98 for these complex emission samples. The parameters examined and reported here include the concentration of S9 and a comparison of Aroclor 1254-induced with uninduced S9 prepared from both rat and hamster livers. The effects of altering these metabolic activation parameters was examined for the organics from diesel car emission, coke oven mains, CSC, and roofing tar emissions.

MATERIALS AND METHODS

Sample Preparation

The coke oven mains sample was collected from a separator located between the gas collector main and the primary coolers within a coke oven battery at Republic Steel in Gadsden, Alabama. The collection and preparation of the remainder of the

samples have been described by Lewtas et al [1981]. The diesel sample was obtained from a Nissan Datsun 220 C.

S9 Preparation

Young adult male CD rats (Charles River Breeding Laboratories, Kingston, NY) and male Syrian golden hamsters (Engel Laboratory Animals, Farmersburg, IN) were used to prepare S9 by the method of Ames et al [1975]. Induced S9 was obtained from rats (200 gm) or hamsters (100 gm) given a single intraperitoneal (IP) injection of Aroclor 1254 (Monsanto, St. Louis, MO) in corn oil at a dose of 500 mg/kg body weight 5 days before sacrifice. Both uninduced and induced S9s were pooled from groups of at least six animals. Protein concentrations were determined by the method of Lowry et al [1951]. These values, expressed in mg/ml, were uninduced rat S9, 30.3; induced rat S9, 26.5; uninduced hamster S9, 27.9; induced hamster S9, 28.9. Each batch of S9 was divided into small aliquots and held at −80°C until use, at which time the protein concentration was adjusted to 25 mg/ml. For assay each S9 was added to a "S9 mix" of buffer and cofactors [Ames et al, 1975] to yield a final concentration of S9 protein of 0.31, 0.63, 1.25, or 2.50 mg in the 0.5 ml aliquot added to each plate.

To insure that the Aroclor-injected animals were induced, each S9 preparation was analyzed for its ability to metabolize benzo(a)pyrene [B(a)P] into eight metabolites—three diols, three quinones, and two phenols—using the method of Nesnow et al [1980]. There was a four-fold increase in total organic-soluble B(a)P metabolites in the induced over the corresponding uninduced S9. The total organic-soluble B(a)P metabolites, expressed in pmoles B(a)P metabolized/min/mg microsomal protein, were uninduced rat S9, 187.9; induced rat S9, 774.0; uninduced hamster S9, 266.8; induced hamster S9, 1,158.0.

Mutagenicity Assay

The *Salmonella*/mammalian microsome mutagenicity assay using strain TA98 was performed as described by Ames et al [1975] with the modifications of Claxton [1981] (minimal level of histidine for growth in the plate agar rather than added to the top agar overlay and incubation of the plates for 72 hr rather than 48 hr at 37°C). Evaluation of the CSC, roofing tar, and diesel samples in five tester strains (TA98, TA100, TA1535, TA1537, TA1538) [Claxton and Huisingh, 1980] resulted in the selection of TA98 for the studies reported here.

Two samples were tested in each experiment with induced and uninduced S9 from either rat or hamster. Experiments were run in duplicate using triplicate plates for each treatment. The time between repeat experiments ranged from 3 to 9 days.

Three doses of each sample plus a control with no sample were tested at the four S9 concentrations for each S9. These levels were selected to be within the range for mutagenic activity but below the toxic level as determined by previous testing. For diesel and CSC, the doses were 30, 100, and 300 μg/plate; and for coke oven mains and roofing tar, they were 5, 50, and 100 μg/plate.

Data Analysis

Student's t-test was used to determine significant differences ($p < 0.01$) between concentrations of S9 at each dose of a sample.

Slopes of the dose-responses (with 95% confidence limits) at each S9 concentration were determined using the non-linear model slope method of Stead et al [1981] by means of the Ames Test Curve Fitting program, a FORTRAN program described by Hasselblad et al [1980].

RESULTS

Figure 1 shows the effects of increasing amounts of S9 on the mutagenicity of the diesel sample. The highest levels of S9 (2.50 mg/plate) resulted in a slight decrease in revertants/plate for most preparations. The diesel extract did not require metabolic activation. In the absence of S9 the values were similar to those with 0.31 mg of S9/plate.

For the coke oven mains (shown in Fig. 2) the mutagenic activity increased with higher concentrations of S9 up to 1.25 mg of S9/plate at the two highest doses of the sample. Aroclor induction resulted in more activity at equivalent S9 levels for both species of animals used. Activity at the lowest S9 concentration used, 0.31 mg/plate, was three- or four-fold that of controls without S9.

The CSC was much more active when the S9 was from Aroclor-induced animals as shown in Figure 3. Uninduced hamster S9 was slightly more effective than uninduced rat S9 in activating the CSC as was the induced hamster compared to the induced rat S9. The highest dose of CSC tested, 300 μg/plate, was the only dose of

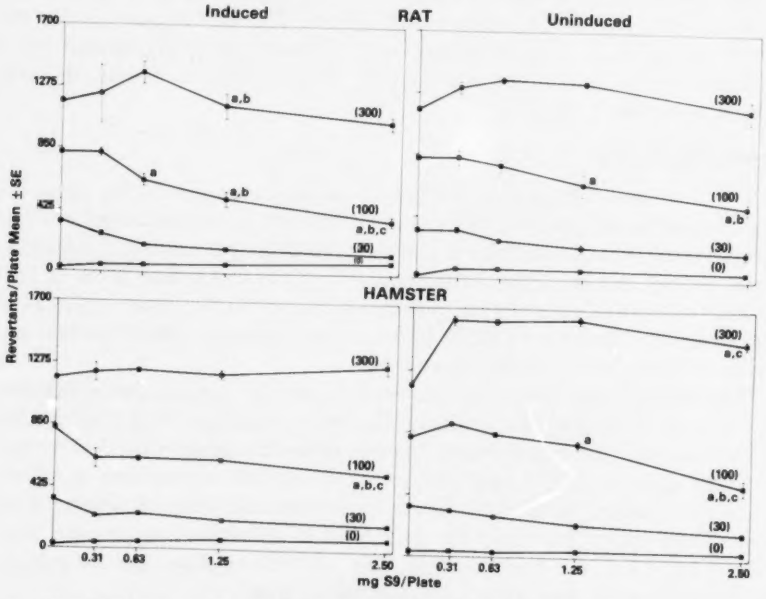

Fig. 1. Diesel (Nissan) sample. Points are mean ± SE of duplicate experiments, each consisting of triplicate plates except for the 0 mg of S9 values, which are the results from one experiment. Numbers in parentheses are μg diesel/plate. a indicates significant difference from the 0.31 mg S9 value at $p < 0.01$. b indicates significant difference from the 0.63 mg S9 values at $p < 0.01$. c indicates significant difference from the 1.25 mg S9 value at $p < 0.01$.

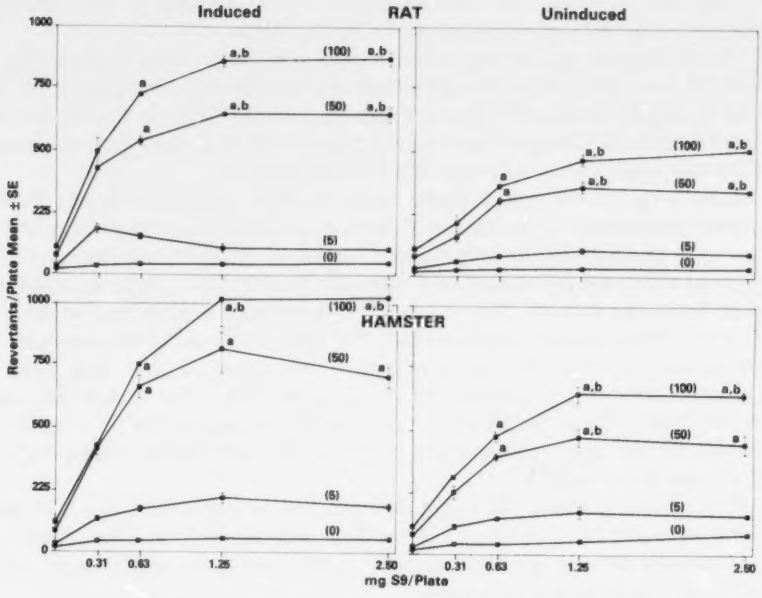

Fig. 2. Coke oven mains sample. Explanation of labeling as in Figure 1.

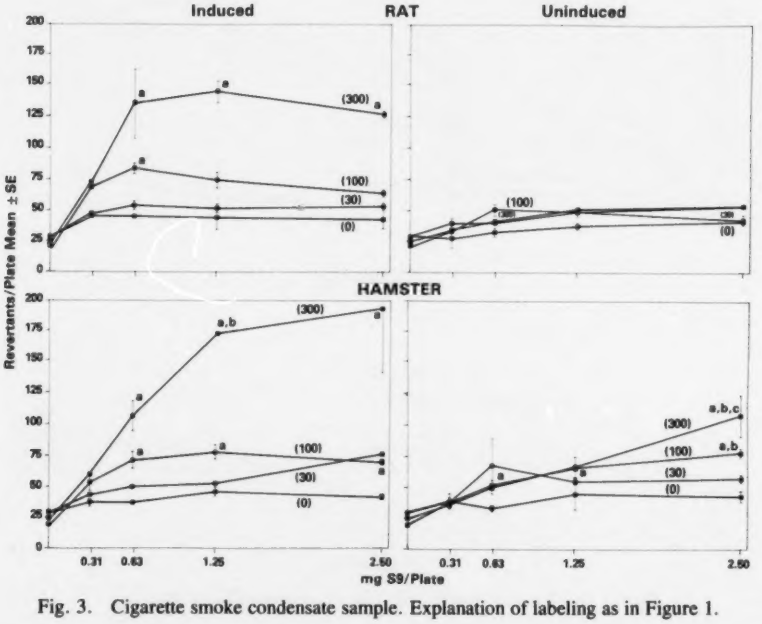

Fig. 3. Cigarette smoke condensate sample. Explanation of labeling as in Figure 1.

CSC that exhibited an S9 effect. There was no mutagenic activity in the absence of S9 at any dose of CSC. The revertants/plate at 300 μg/plate of CSC increased significantly as the S9 was increased up to 1.25 mg S9/plate.

The roofing tar was the least active sample regardless of the S9 used (Fig. 4). Induced S9 was again more effective than the corresponding uninduced S9 with induced rat slightly more active than hamster. As with the CSC, no activity was seen in the absence of S9. The revertants/plate increased with increasing concentrations of S9 at the two highest doses of sample (50 and 100 μg/plate).

Table I reports the model slopes (with the 95% confidence limits) for each experiment determined by the method of Stead et al [1981]. Since these samples were assayed only at doses in the initial linear portion of the dose-response curve, the model slope values are not significantly different from the linear slopes as determined by linear regression analysis. The slopes for the diesel sample were very similar when rat liver S9, either induced or uninduced, was used as the metabolic activator. For uninduced hamster liver S9, the slopes were consistently higher than those for uninduced rat liver S9. In general, the diesel sample was more mutagenic, with a higher slope value, the lower the dose of S9. The narrow range of the 95% confidence limits indicates the high reproducibility observed between replicate plates when the diesel samples were assayed.

For coke oven mains, the slopes with induced S9 were about two-fold higher than the corresponding uninduced S9 for both hamster and rat. The slopes with induced hamster S9 were generally slightly higher than those with induced rat S9. The 95% confidence limits were narrow.

The slopes for CSC were the lowest of the four mixtures tested. Again, slopes were higher when induced S9 was used, and those for induced hamster S9 were

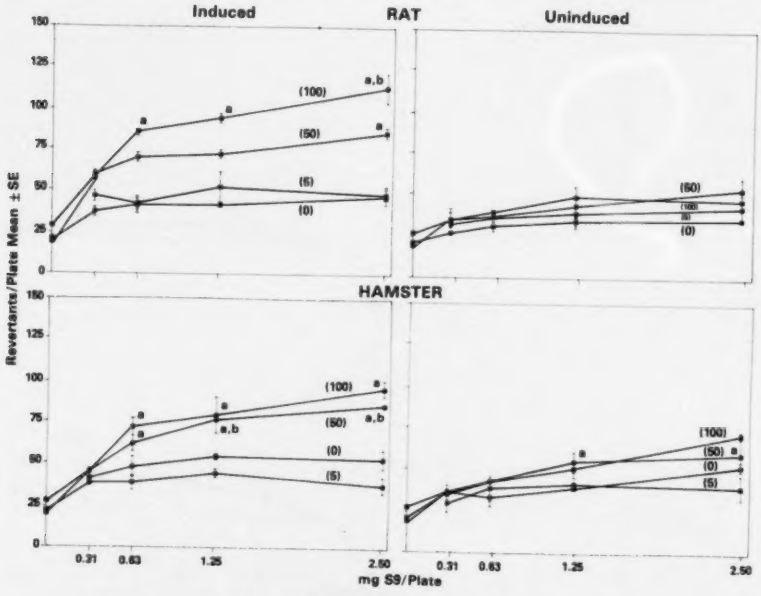

Fig. 4. Roofing tar sample. Explanation of labeling as in Figure 1.

TABLE I. Model Slopes (With 95% Confidence Limits) for Duplicate Experiments

			Rat					Hamster			
			Induced		Uninduced			Induced		Uninduced	
Sample	Exp.	S9 mg/plate	Model slope	95% Confidence limits	Model slope	95% Confidence limits	S9 mg/plate	Model slope	95% Confidence limits	Model slope	95% Confidence limits
Diesel: Nissan	1	0.31	4.82	4.76, 4.87	4.19	4.10, 4.29	0.31	4.13	4.08, 4.18	5.56	5.48, 5.64
		0.63	4.27	4.21, 4.32	4.53	4.43, 4.63	0.63	4.06	3.92, 4.19	5.49	5.42, 5.58
		1.25	3.93	3.79, 4.07	4.41	4.29, 4.52	1.25	4.03	3.98, 4.09	5.34	5.26, 5.43
		2.50	3.06	2.99, 3.14	3.52	3.43, 3.62	2.50	4.19	4.09, 4.30	4.92	4.64, 5.22
	2	0.31	4.76	4.68, 4.85	4.45	4.38, 4.51	0.31	3.86	3.81, 3.91	5.32	5.24, 5.39
		0.63	4.80	4.72, 4.89	4.78	4.73, 4.84	0.63	3.95	3.83, 4.08	5.34	5.29, 5.40
		1.25	3.51	3.47, 3.55	4.38	4.29, 4.46	1.25	3.78	3.69, 3.88	5.52	5.44, 5.61
		2.50	3.43	3.38, 3.49	4.01	3.93, 4.09	2.50	3.89	3.77, 4.02	4.59	4.51, 4.69
Coke oven mains	1	0.33[a]	4.35	4.16, 4.55	2.23	2.11, 2.35	0.31	4.23	4.11, 4.35	2.82	2.66, 2.99
		0.66[b]	6.92	6.77, 7.07	3.78	3.69, 3.86	0.63	7.59	7.44, 7.77	4.67	4.58, 4.77
		1.32[c]	8.55	8.41, 8.69	4.41	4.29, 4.54	1.25	11.62	11.45, 11.78	6.09	5.94, 6.24
		2.65[d]	7.99	—	4.84	4.33, 5.42	2.50	10.58	10.38, 10.77	5.54	5.33, 5.75
	2	0.31	5.15	4.78, 5.55	1.58	1.41, 1.77	0.31	4.36	4.24, 4.48	2.65	2.49, 2.81
		0.63	7.21	7.01, 7.42	3.45	3.32, 3.59	0.63	7.95	7.84, 8.06	4.40	4.29, 4.51
		1.25	9.15	9.03, 9.26	4.72	4.45, 5.00	1.25	8.78	8.58, 8.99	6.14	5.93, 6.36
		2.50	8.69	8.58, 8.82	4.82	4.66, 4.99	2.50	9.17	9.01, 9.34	5.74	5.52, 5.97

continued

TABLE I. Model Slopes (With 95% Confidence Limits) for Duplicate Experiments (Continued)

		Rat					Hamster			
		Induced		Uninduced			Induced		Uninduced	
Sample	Exp.	S9 mg/plate	Model slope	95% Confidence limits	Model slope	95% Confidence limits	S9 mg/plate	Model slope	95% Confidence limits	Model slope	95% Confidence limits
Cigarette smoke condensate	1	0.31	0.09	0.05, 0.21	0.04	0.01, 0.39	0.31	0.08	0.05, 0.14	0.01	0.00, 1.20
		0.63	0.22	0.17, 0.28	0.06	0.03, 0.09	0.63	0.28	0.23, 0.34	0.07	0.01, 0.53
		1.25	0.29	0.23, 0.37	0.05	0.02, 0.19	1.25	0.43	0.38, 0.49	0.10	0.06, 0.16
		2.50	0.25	0.21, 0.31	0.05	0.01, 0.27	2.50	0.35	0.32, 0.38	0.18	0.14, 0.24
	2	0.31	0.11	0.08, 0.16	0.03	0.01, 0.07	0.31	0.08	0.05, 0.14	0.00	0.00, 0.00
		0.63	0.39	0.35, 0.46	0.03	0.01, 0.17	0.63	0.19	0.11, 0.35	0.17	0.12, 0.25
		1.25	0.39	0.25, 0.64	0.04	0.00, 1.31	1.25	0.41	0.34, 0.49	0.06	0.03, 0.12
		2.50	0.29	0.27, 0.33	0.05	0.01, 0.16	2.50	0.57	—	0.26	0.21, 0.31
Roofing tar	1	0.33[a]	0.24	0.08, 0.73	0.10	0.05, 0.19	0.31	0.09	0.02, 0.51	0.06	0.02, 0.17
		0.66[b]	0.49	0.34, 0.69	0.11	0.07, 0.18	0.63	0.37	0.23, 0.59	0.16	0.10, 0.25
		1.32[c]	0.45	0.33, 0.60	0.17	0.07, 0.42	1.25	0.33	0.23, 0.47	0.13	0.03, 0.68
		2.65[d]	0.00	0.00, 0.00	0.19	0.12, 0.29	2.50	0.64	0.55, 0.74	0.22	0.09, 0.52
	2	0.31	0.23	0.17, 0.30	0.15	0.04, 0.48	0.31	0.06	0.01, 0.31	0.00	0.00, 0.00
		0.63	0.46	0.35, 0.62	0.05	0.01, 0.34	0.63	0.29	0.19, 0.43	0.09	0.04, 0.22
		1.25	0.56	0.37, 0.87	0.12	0.03, 0.49	1.25	0.43	0.23, 0.79	0.18	0.08, 0.42
		2.50	0.80	0.63, 1.03	0.14	0.08, 0.26	2.50	0.57	0.49, 0.72	0.97	0.88, 1.08

[a]For this experiment, concentration of uninduced rat S9 = 0.38 mg/plate.
[b]For this experiment, concentration of uninduced rat S9 = 0.76 mg/plate.
[c]For this experiment, concentration of uninduced rat S9 = 1.51 mg/plate.
[d]For this experiment, concentration of uninduced rat S9 = 3.03 mg/plate.

slightly higher than those with induced rat S9. A wider range in 95% confidence limits was observed for the CSC compared to the diesel and coke oven mains.

Experiments with roofing tar gave results similar to those with CSC with induced S9 model slopes being higher than with uninduced. The induced rat S9 slopes, however, were slightly higher than those with induced hamster S9. Overall, the slopes were only slightly higher than those for CSC. The 95% confidence limits were similar to those for CSC.

When Aroclor-induced rat liver S9 was used with the complex mixtures that required S9, the highest model slopes were generally found at the 1.25 mg S9/plate level. In the case of induced hamster S9, 2.50 mg/plate often resulted in slightly higher model slopes. When uninduced S9, either hamster or rat, was tested, the highest slopes were at the higher concentration of S9. The exception to these observations was the diesel where the lower doses 0.31–0.63 mg/plate gave the highest slope. With this direct-acting sample, increasing S9 levels resulted in decreasing mutagenic activity.

The mutagenic activity of these four complex emission samples with rat and hamster S9 is compared on a relative basis in Table II using the optimum S9 concentration (1.25 mg/plate). In order to compare the relative potency across different bioassays, the slopes are all normalized to one sample, the coke oven mains. Table II also contains relative potency values for the same samples as previously reported [Nesnow and Lewtas, 1981; Albert et al, 1983] from the Ames *S typhimurium* TA98 (+S9), mouse lymphoma, and mouse skin tumor initiation assays. The relative potency of the rat and hamster S9 activation systems in the Ames TA98 assay are nearly identical based on the data presented here and are two- to four-fold less than earlier reported. Alteration of the S9 activation, however, did not improve the correlation of the relative potencies of these samples in the Ames assay compared to the relative mouse skin tumor initiation activity.

TABLE II. Relative Potencies of Emission Samples in Several Bioassay Systems

Sample	Mouse skin tumor initiation[a]	Mouse lymphoma (+MA)[b]	Ames *Salmonella*: TA98 (+MA)		
			Rat S9[c]	Rat S9[d]	Hamster S9[d]
Coke oven mains	1.0	1.0	1.0	1.0	1.0
Roofing tar	0.13	0.63	0.13	0.06	0.04
Cigarette smoke condensate	0.00075	0.029	0.085	0.04	0.05
Diesel:Nissan	0.18	0.11	1.9	0.43	0.39

[a]Values based on papilloma/mouse at 1 mg. Emission samples at five dose levels were applied in spectral-grade acetone to shaved skin of male and female SENCAR mice. Papillomas greater than 2 mm persisting for 1 wk or longer were scored [Slaga et al, 1981]. Data recalculated from Table III in Albert et al [1983] setting the value for coke oven mains at 1.0.

[b]Mutation frequency (thymidine kinase [TK] mutants/10^6surviving cells)/μg/ml. The L5178Y mouse lymphoma TK forward mutation assay was run with ten doses of each emission sample. The mutation frequency was expressed as the ratio of mutant cells to surviving cells [Mitchell et al, 1981]. Data recalculated from Table II in Albert et al [1983] setting the values for coke oven mains at 1.0.

[c]Linear slope (revertants/μg). Data recalculated from Table II in Albert et al [1983] setting the value for coke oven mains at 1.0. S9 from Aroclor-induced animals.

[d]Linear slope (calculated by the Ames Test Curve Fitting program [Hasselblad et al, 1980]) at S9 concentration of 1.25 mg/plate. S9 from Aroclor-induced animals.

DISCUSSION

This study has confirmed earlier reports [Claxton and Huisingh, 1980; Claxton, 1981] that the extractable organics from diesel particle emissions are direct-acting in the Ames *S typhimurium* mutagenesis assay. Nitrated polynuclear aromatic hydrocarbons have recently been detected [Schuetzle et al, 1981] and quantitated [Nishioka et al, 1983] in these samples and appear to account for a significant portion of the "direct-acting" activity. Studies on the activation of nitrated polynuclear aromatic hydrocarbons in *S typhimurium* have shown that nitroreductases present in the bacteria are essential to expression of the mutagenicity and that, in some cases (eg, dinitropyrenes), the addition of liver S9 activation has decreased the mutagenicity, whereas in other cases (eg, 6-NO_2-BaP), S9 has increased the mutagenicity [Tokiwa et al, 1981].

The other three complex organic samples either absolutely required S9 activation (roofing tar and CSC) or required S9 activation for maximum activity (coke oven mains). In every case the induced S9 produced more revertants/plate and a higher mutagenic activity as measured by the slope of the dose-response. The induced S9 had a significantly higher mixed function oxidase activity as measured by formation of individual and total organic soluble B(a)P metabolites. Although all three of these samples contain B(a)P and other polynuclear aromatic hydrocarbons (PNAs), fractionation and mutagenicity studies showed that more mutagenic activity is found in either the organic bases (coke oven mains and CSC) or polar neutral compounds (roofing tar) [Austin et al, 1985]. The standard S9 concentration (approximately 1.25 mg protein/plate) also resulted in the maximum mutagenic activity for these samples.

The hamster S9 activation gave significantly higher mutagenic activities for the coke oven and CSC samples. These two samples both exhibited major mutagenic activity in the organic base fraction that contains aromatic amines and nitrogen heterocycles [Austin et al, 1985]. Aroclor-induced hamster S9 has been shown to be more effective than induced rat S9 in activating aromatic amines [Raineri et al, 1981]. Since the induced hamster S9 was equal to or better than the induced rat S9 for optimal activity, it may be a better choice for screening such complex emission samples.

Changing some metabolic activation parameters of the Ames test did not significantly alter the relative potency of the four emission samples in the Ames assay. The CSC was two to three orders of magnitude more mutagenic in both the microbial and the mouse lymphoma mutagenesis bioassays than observed in the mouse skin tumor initiation assay and human lung cancer unit risk estimates [Albert et al, 1983]. These results suggest that the differences observed between the relative mutagenic activity of these four emissions in the mammalian cell and microbial assays do not appear to be due to a lack of optimization of the S9 system, but rather may be inherent in the different response of the indicator cell (eg. *Salmonella*) to different classes of chemicals.

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